Parathyroid hormone-related protein promotes inflammation in the kidney with an obstructed ureter

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Parathyroid hormone-related protein (PTHrP) promotes fibrogenesis in the acutely damaged kidney. Considering the relation between fibrosis and inflammation, we studied transgenic mice that overexpress PTHrP in the proximal tubule. When unilateral ureteric obstruction was induced in these transgenic mice, we found that they had more renal tubulointerstitial damage, leukocyte influx, and expression of proinflammatory factors than their control littermates. Reversal of PTHrP constitutive overexpression in these transgenic mice or treatment of control mice with the PTHrP antagonist (7–34) decreased this inflammatory response. Losartan, which abolished obstruction-induced endogenous PTHrP upregulation, also decreased the latter response but less effectively in transgenic mice. The PTHrP fragment (1–36) induced nuclear factor-κB (NF-κB) activation and proinflammatory cytokine overexpression in mouse cortical tubule cells in culture as well as migration of the macrophage cell line Raw 264.7. All these effects were decreased by PTHrP (7–34) and NF-κB or extracellular signal-regulated kinase (ERK) activation inhibitors. Our findings suggest a critical role of PTHrP in the renal inflammatory process that results from ureteral obstruction and indicate that ERK-mediated NF-κB activation seems to be an important mechanism whereby PTHrP triggers renal inflammation.

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Parathyroid hormone (PTH)-related protein (PTHrP) is upregulated in various experimental nephropathies.[1] PTHrP overexpression correlates with the development of proteinuria in both diabetic mice and rats with tubulointerstitial damage after protein overload.[2,3] Moreover, recent studies indicate that PTHrP might contribute to tubular damage, renal function deterioration, and fibrosis after nephrotoxic injury in rodents.[4–6]

Inflammation plays a key role in progressive renal scarring and fibrogenesis in various kidney diseases.[7–10] Nuclear factor-κB (NF-κB)-related cytokines appear to have an important role in renal injury-associated inflammation.[11–14] This factor commonly consists of a heterodimer of one p50 subunit and one p65 subunit—the former being transcriptionally repressive—that translocates to the nucleus upon activation to regulate gene transcription.[13,15,16] In fact, angiotensin (Ang) II receptor antagonists confer renal protection apparently through inhibition of NF-κB-dependent proinflammatory pathways.[12,17] However, the putative factors triggering renal inflammation are not fully understood.

PTHrP displays proinflammatory features in various pathophysiological settings.[18] Recently, an increased interstitial macrophage influx was found to be associated with PTHrP overexpression in mice with folic acid-induced nephrotoxicity.[6] In this study, we explored whether PTHrP might be a key cytokine-inducing inflammation in the injured kidney.[19,20] We examined the functional consequences of chronic PTHrP upregulation in mouse kidney after unilateral ureteral obstruction (UUO), characterized by an early renal inflammatory response.[8,19–21] Furthermore, we assessed the putative intracellular pathways underlying the proinflammatory effects of PTHrP on renal tubuloepithelial cells.

RESULTS

Tubulointerstitial alterations occur associated with PTHrP upregulation in the obstructed mouse kidney

Endogenous mouse PTHrP mRNA levels increased similarly in the obstructed kidneys of control and PTHrP-overexpressing transgenic (PTHrP-TG) mice after UUO over corresponding levels in sham-operated mice (Figure 1a). By

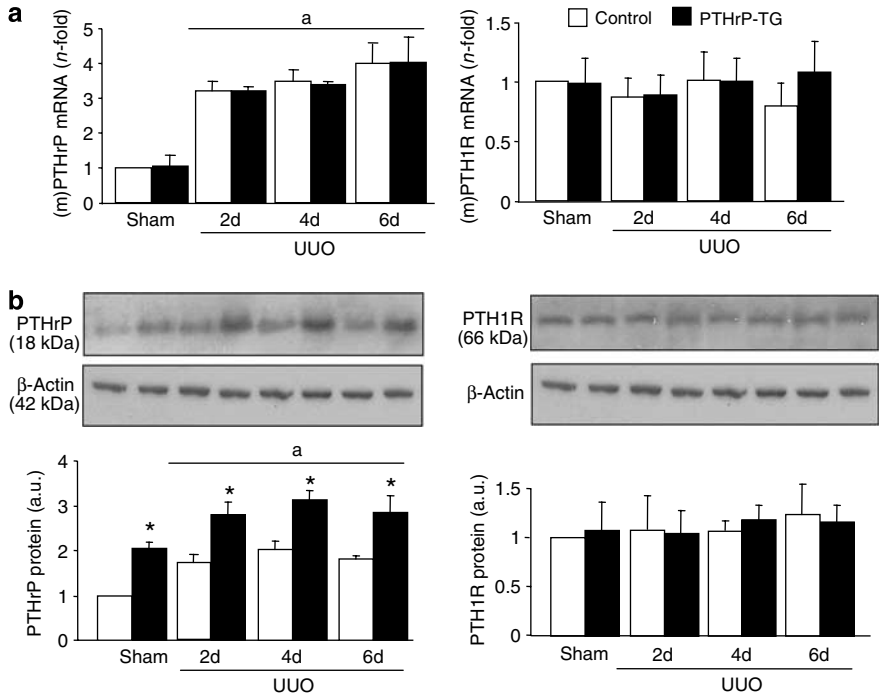

Figure 1 | PTHrP and PTH1R expression in the obstructed kidney from PTHrP-TG mice and their control littermates. In the kidney of sham-operated mice or the obstructed kidney for 2–6 days, we analyzed endogenous mouse (m)PTHrP gene expression (by real-time PCR) (**a**); PTHrP protein (by western blot, using antibody C6 recognizing both endogenous PTHrP and the human PTHrP transgene) (**b**); and the PTH1R gene (by real-time PCR) and protein (by western blot) expression (**a**, **b**). Representative autoradiograms are also shown in (**b**). Experimental values are mean ± s.e.m. of 4–6 mice per group at each time period. All values were normalized against corresponding sham control. $^{*}P<0.01$ vs corresponding value in control mice; $^{a}P<0.01$ vs corresponding value in sham-operated mice. AU, arbitrary units.

using PTHrP antiserum C6, recognizing the conserved 107-111 epitope in intact PTHrP in mice and humans,[3,6,22] PTHrP levels were twofold higher in the unobstructed kidney of PTHrP-TG mice than in that of control littermates and likewise increased after UUO (Figure 1b). A single band of an apparent molecular weight of 18 kDa was detected with this antiserum, corresponding to the single full-length PTHrP isoform in mice, as reported previously.[3,6] Meanwhile, PTH1 receptor (PTH1R) mRNA and protein levels remained unchanged within the same time period in these mice (Figure 1a and b).

Tubular atrophy and interstitial fibrotic areas in the obstructed kidneys occurred earlier, and the former was higher throughout the study in PTHrP-TG mice than in control littermates (Figure 2a). Both sham-operated kidneys and contralateral kidneys (data not shown) from PTHrP-TG mice showed no structural alterations and had a similar scarce number of interstitial leukocytes as in control littermates (Figure 2a and b). Starting 2 days after obstruction, a dramatic increase of the latter occurred in both types of mice, mainly in PTHrP-TG mice (Figure 2b).

PTHrP overexpression in the obstructed mouse kidney is related to increased proinflammatory factors

Various proinflammatory and chemotactic cytokines and cell adhesion molecules are involved in the inflammatory response after UUO.[8,14,19,21,23] In the unobstructed kidneys, we observed a higher (mRNA and/or protein) expression of monocyte chemoattractant protein (MCP)-1, MCP-1 receptor CCR2 (chemokine (C–C motif) receptor 2), RANTES (regulated upon activation, normal T-cell expressed, and secreted), and interleukin (IL)-6 in PTHrP-TG mice than in control mice (Figure 3). After UUO, there was a stepwise increase of the three former factors in the obstructed kidneys of control mice. This increase of MCP-1 protein and RANTES gene expression was higher in PTHrP-TG mice throughout the study (Figure 3a–c). Meanwhile, CCR2 gene expression reached its maximum earlier in the PTHrP-TG mice than in control littermates following obstruction (Figure 3a–c). A sustained increase of intercellular adhesion molecule (ICAM)-1 mRNA levels also occurred in the obstructed kidneys of the former animals (Figure 3d). In contrast, an early and transient increase of IL-6 gene expression was observed in these kidneys, which was more dramatic in PTHrP-TG mice (Figure 3e). Moreover, consistent with these findings and previous data,[14,19] we found elevated levels of NF-κB activity in the obstructed mouse kidney extracts, which were significantly higher in PTHrP-TG mice (Figure 4).

Effects of pharmacological suppression of PTHrP on inflammation in the obstructed mouse kidney

To confirm that PTHrP was responsible for the proinflammatory changes in the obstructed mouse kidney, we used several experimental maneuvers. First, PTHrP-TG mice were administered doxycycline (20 mg l^{-1} in drinking water;

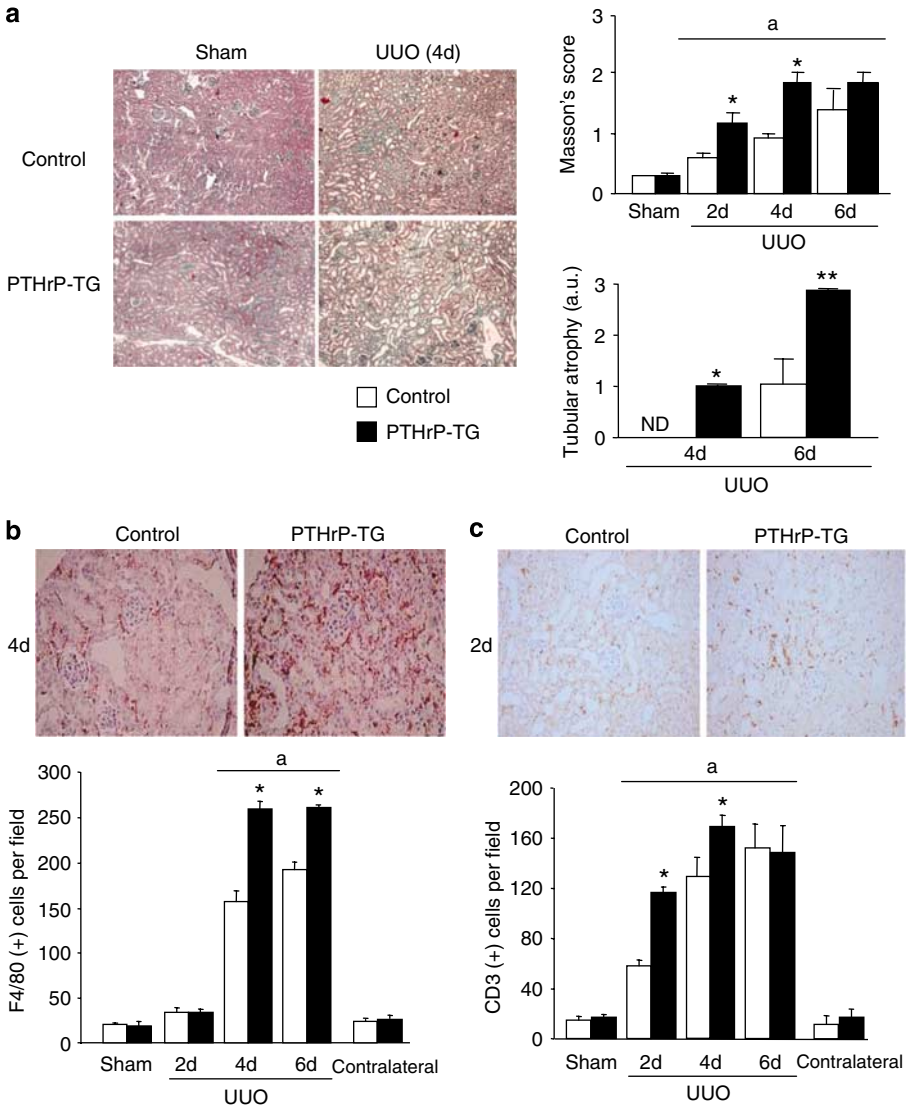

Figure 2 | Evolution of renal lesions, as well as inflammatory cell infiltration, up to 6 days in the obstructed kidney of control and PTHrP-TG mice. Renal lesions (assessed by Masson's staining, original magnification $\times 100$) (**a**), the number of renal interstitial macrophages (determined by F4/80 staining) (**b**), and T lymphocytes (identified by CD3 positivity) (**c**) (original magnification $\times 200$) in the kidney of representative control and PTHrP-TG mice at the indicated days of obstruction or sham operation are shown. Score values are mean ± s.e.m. of 4–6 animals per group at each time. These values corresponding to sham-operated and contralateral kidneys were unchanged throughout the period of study, and thus those for the different times in each group were pooled. *$P<0.01$ vs corresponding value in control mice. [a]$P<0.01$ vs corresponding value in sham-operated mice. ND, not detected.

a lower dose than that exhibiting anti-inflammatory effects[24,25]) for 1 week before and during obstruction (4 days) to reverse constitutive PTHrP overexpression.[6,26] As expected, we did not find any effect of this treatment on the number of interstitial macrophages (by F4/80 staining) in the obstructed kidney of control mice; these values were 160 ± 6 or 166 ± 18, with or without doxycycline treatment ($n = 4$). In contrast, in the obstructed kidneys of PTHrP-TG mice, doxycycline administration decreased NF-κB activity (Figure 4), as well as CCR2, RANTES, and ICAM mRNA levels, to that found in control mice (Figure 5). This indicates that the higher expression of these factors in PTHrP-TG mice is accounted for by the PTHrP transgene.

Next, we treated control mice with PTHrP (7–34), a PTH1R antagonist, before and after UUO.[6,27] This treatment was associated with a significant decrease (about 40–50%) in interstitial leukocytes and MCP-1 and RANTES expression in the obstructed kidneys (Figure 6a and b). The AT_1 receptor antagonist losartan was previously shown to abolish PTHrP upregulation elicited by nephrotoxic acute renal injury in rats.[5] This antagonist, in contrast to an AT_2 antagonist, also prevented the PTHrP mRNA overexpression and decreased in part the renal inflammatory response in the obstructed kidneys of control mice (Figure 7a–c). Of note, losartan was more efficient in this regard in the latter mice than in PTHrP-TG mice, in which it only inhibited by 25% interstitial

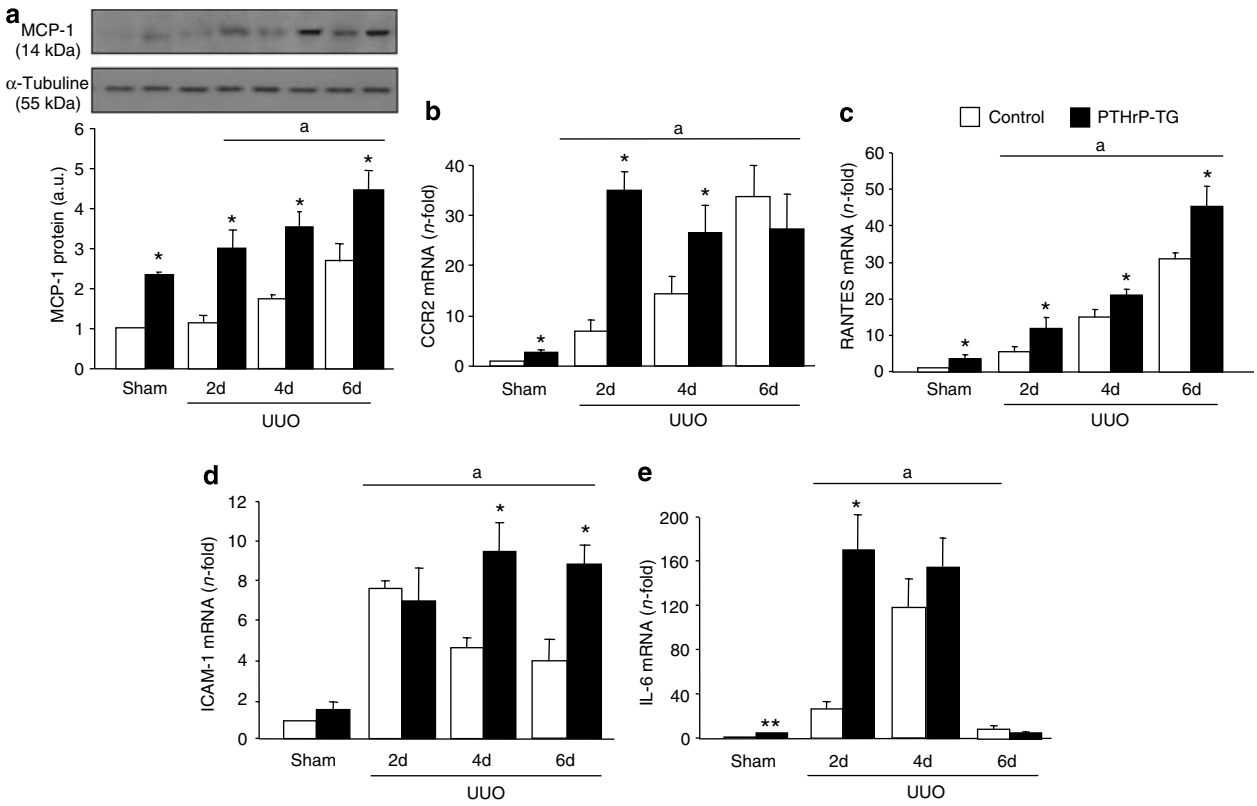

Figure 3 | Changes in several proinflammatory factors in the obstructed kidney of control and PTHrP-TG mice. Protein expression of MCP-1 (**a**) (representative autoradiogram of western blot analysis is shown) and gene expression of CCR2 (**b**) RANTES (**c**), ICAM-1 (**d**), and IL-6 (**e**) (determined by real time PCR) in the kidney of both types of mice at 2-6 days after UUO or sham operation. Experimental values are mean ± s.e.m. of 4–6 animals per group at each time. *$P<001$ vs corresponding value in control mice. [a]$P<0.01$ vs corresponding value in sham-operated mice. AU, arbitrary units.

macrophage infiltration (compared to 60% in control mice), and it did not significantly affect renal MCP-1 expression (Figure 7b and c).

Role of NF-κB and ERK activation on the PTHrP-induced proinflammatory mechanisms in mouse cortical tubuloepithelial (MCT) cells

Tubular cells produce proinflammatory factors to promote leukocyte influx after renal scarring.[8] Thus, the next experiments were performed using these cells *in vitro* to assess the molecular mechanisms whereby PTHrP induces inflammation in the obstructed mouse kidney. Consistent with our *in vivo* findings, PTHrP (1–36), at 100 nM, maximally (at 20–30 min) increased NF-κB–DNA binding, which was decreased by anti-p50 or anti-p65 antibodies, in MCT cell nuclear extracts (Figure 8a). Moreover, this peptide dose dependently induced p65 translocation to the nucleus in MCT cells at 30 min, decreasing thereafter (Figure 9a and b). Furthermore, PTHrP (1–36), within the same concentration range and time frame, induced MCP-1 and RANTES gene expression in these cells, an effect abolished by PTHrP (7–34) (Figure 10a and b). Pretreatment with several NF-κB inhibitors, which prevent IκB degradation,[28,29] dramatically inhibited the effect elicited by PTHrP (1–36) on the gene expression of various proinflammatory factors in these cells (Figure 11). NF-κB activation can occur associated with ERK phosphorylation in renal cells.[30,31] We presently found that ERK 1/2 was rapidly and transiently activated by PTHrP (1–36), at 100 nM, in MCT cells (Figure 8b). Moreover, the ERK 1/2 kinase inhibitor U0126 significantly reduced NF-κB activation (Figures 8a and 9a) and also overexpression of the aforementioned proinflammatory factors by PTHrP (1–36) in these cells (Figure 11). Neither of these inhibitors significantly affected the expression of these factors in MCT cells (data not shown).

PTHrP (1–36) stimulates migration of mouse macrophages Raw 264.7

The medium conditioned by PTHrP (1–36)-treated MCT cells was found to induce Raw 264.7 cell migration (Figure 12a). This was prevented by pretreatment with either PTHrP (7–34) or several ERK or NF-κB inhibitors (Figure 12a). The addition of an MCP-1 antibody to the MCT cell-conditioned medium after PTHrP (1–36) stimulation partly inhibited its chemoattractant activity (Figure 12a). Interestingly, the addition of PTHrP (7–34) or an N-terminal PTHrP antiserum to this medium also neutralized in part this activity (Figure 12a), suggesting that PTHrP (1–36) might directly stimulate Raw 264.7 cell migration. In fact, it was proven to do so when we exposed the latter cells to PTHrP (1–36) in serum-free

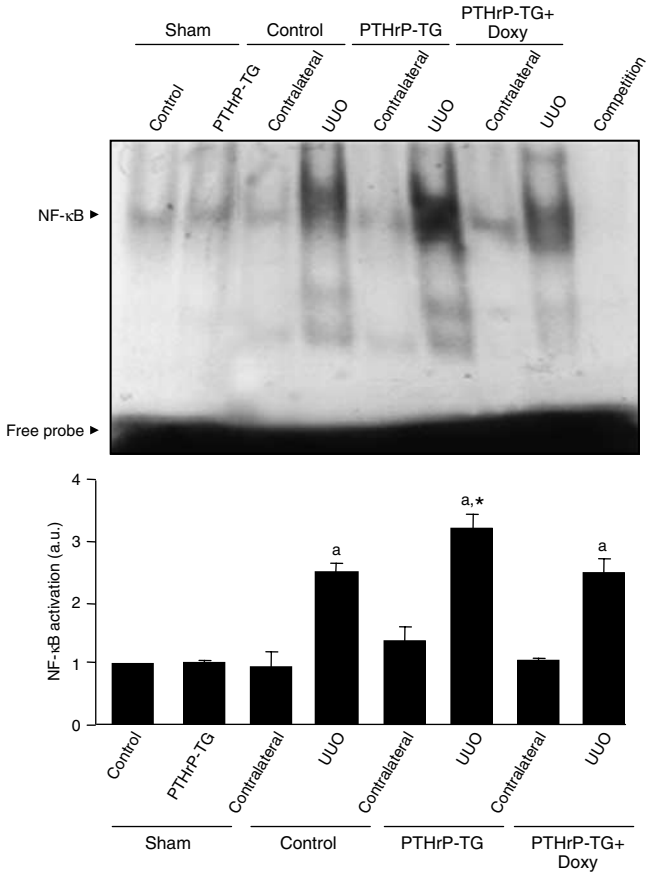

Figure 4 | Changes in NFκB activation in the obstructed mouse kidney. NF-κB activation was determined by EMSA in total kidney extracts from PTHrP-TG and control mice at day 4 after UUO or sham operation, as described in the text. Some PTHrP-TG mice were pretreated with doxycycline (doxy) (20 mg l^{-1} in the drinking water) for 1 week before and during obstruction (4 days) to suppress PTHrP overexpression. A representative EMSA is shown (upper panel). As control for specificity, cell extracts were pre-incubated with a 100-fold excess of unlabeled NF-κB oligonucleotide for 10 min at 4 °C before adding the labeled probe. Relative densitometric values as mean ± s.e.m. over that of sham-operated control from four animals per group are shown (lower panel). $^{a}P<0.01$ vs corresponding sham or contralateral kidney values. $^{*}P<0.01$ vs corresponding control and doxy-treated values. AU, arbitrary units.

medium (Figure 12b). A high dose (200 μg ml^{-1}) of doxycycline did not affect this peptide stimulation of macrophage migration, which was 500 ± 100 or 544 ± 45%, in the presence or absence of the drug, respectively, compared to basal value (100%) ($n=4$), supporting further our aforementioned *in vivo* findings in doxycycline-treated mice. In contrast, this effect of PTHrP (1–36) was abolished by both PTHrP (7–34) and an MCP-1 antibody (Figure 12b). PTHrP (1–36) also directly increased MCP-1 mRNA expression in Raw 264.7 cells, and this was abrogated by PTHrP (7–34) (Figure 12c).

DISCUSSION

In this study, PTHrP was found to be upregulated in the obstructed mouse kidney, even in PTHrP-TG mice, further supporting that the PTHrP transgene does not seem to affect endogenous PTHrP gene expression in mice.[26] In contrast to previous observations in ischemic or nephrotoxic renal injury,[1,5,6,26] PTH1R was not downregulated after UUO in mice. Interestingly, an upregulation of both PTHrP and PTH1R was recently observed in the kidney of diabetic mice.[3] The reasons for the different response of PTH1R to various renal injuries are presently unknown.

In the obstructed mouse kidney, PTHrP upregulation was associated with renal fibrosis and inflammation, consistent with previous findings in another model of acute renal injury.[6] After UUO, we observed herein that the increase of several proinflammatory factors (namely, MCP-1, RANTES, and ICAM-1) was of greater magnitude, associated with higher tubular injury, in PTHrP-TG mice than in control littermates at the end of study. In this regard, a relationship between tubular injury and renal inflammation has been reported.[8,32,33]

The kidneys of PTHrP-TG mice showed no morphologic or functional alterations in basal conditions.[3,6] In this study, the unobstructed kidney of these mice had a higher expression of various proinflammatory mediators than that of their control littermates, although leukocyte infiltration was similar in both types of mice. In this regard, we recently

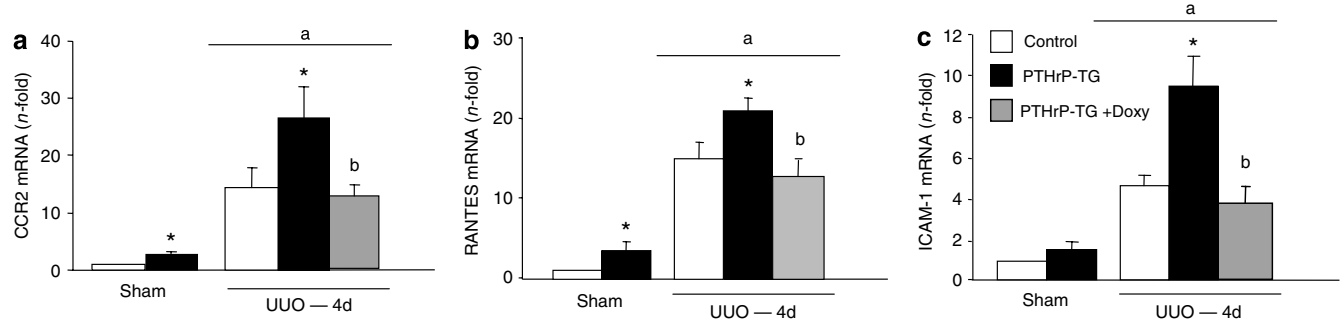

Figure 5 | Effect of doxycyclin on several proinflammatory factors in the obstructed kidney of PTHrP-TG mice. Treatment with doxycycline, as described in the legend to Figure 4, reduced CCR2 (**a**), RANTES (**b**), and ICAM-1 (**c**) gene upregulation (by real-time PCR) in PTHrP-TG mice to the corresponding levels in control mice at day 4 after UUO. Experimental values are mean ± s.e.m. of 4–6 animals per group at each time. $^{*}P<0.01$ vs corresponding value in control mice; $^{a}P<0.01$ vs corresponding value in sham-operated mice; $^{b}P<0.01$ vs corresponding PTHrP-TG value.

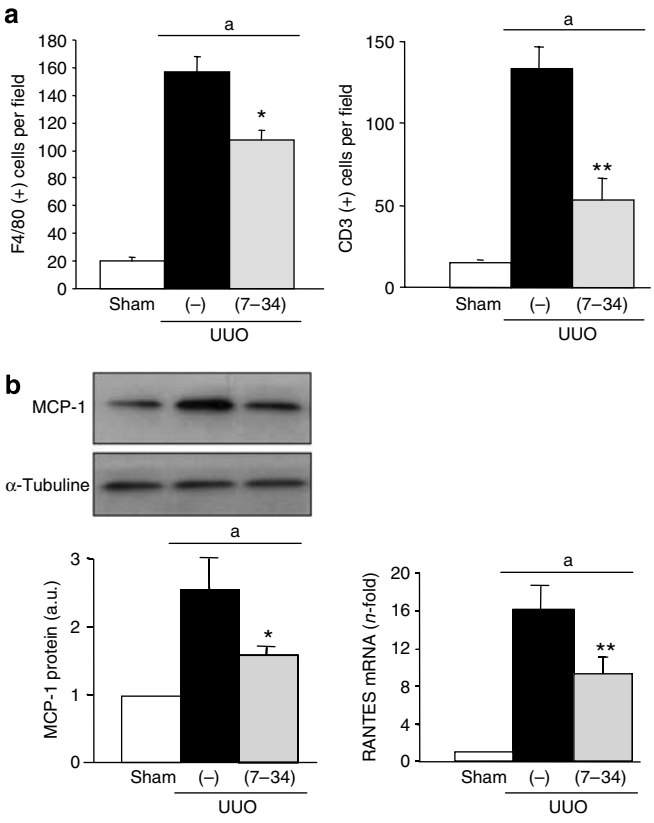

Figure 6 | Effect of a PTH1R antagonist on various proinflammatory alterations in the obstructed kidney of control mice. The number of renal interstitial macrophages (identified by F4/80 staining) and T lymphocytes (assessed by CD3 positivity) (**a**), as well as MCP-1 protein expression (representative autoradiogram of western blot analysis is shown) and RANTES mRNA expression (by real-time PCR) (**b**) were evaluated in the kidney of control mice at day 4 after UUO or sham operation. PTHrP (7–34) was administered to a group of mice at 35 μg per animal per day, 1 day before and during obstruction for 4 days. Experimental values are mean ± s.e.m. of at least four animals per group. $^{a}P<0.01$ vs value in sham-operated mice. $^{*}P<0.01$; $^{**}P<0.05$ vs value in untreated mice. AU, arbitrary units.

reported that PTHrP-TG mice developed higher renal hypertrophy and proteinuria than control mice after diabetes induction.[3] These findings suggest that constitutive PTHrP overexpression in mice might trigger counterregulating mechanisms, such as nitric oxide production,[34,35] which would prevent renal damage in the absence of specific kidney insults. Confirming this hypothesis requires further studies. In any event, current data point to PTHrP as a factor that could accelerate an injury-dependent development of renal inflammation and fibrosis.

Doxycycline-induced reversal of the PTHrP transgene in the obstructed kidney of PTHrP-TG mice restored the response of proinflammatory factors to corresponding levels in control mice. Moreover, in the latter mice, pretreatment with PTHrP (7–34) reversed a significant part of several proinflammatory effects induced by UUO. This suggests a partial efficiency of this antagonist at the dose used and/or that PTHrP might affect inflammation through an intracrine mechanism. However, this is unlikely, as suppression of PTHrP upregulation by losartan was equally efficient as the PTH1R antagonist in inhibiting inflammation in the obstructed mouse kidney. Interestingly, losartan was less efficient in this regard in PTHrP-TG mice, suggesting that PTHrP might recapitulate in part the proinflammatory actions of angiotensin II in this setting.[19,23,36]

NF-κB activation is known to be a key factor in the renal inflammatory response to kidney injury.[11–14,19,37] Consistent with the observed changes in various NF-κB-dependent factors, we found here an increased NF-κB activity associated with PTHrP overexpression in the obstructed mouse kidney. Furthermore, our present *in vitro* data show that induction of MCP-1, RANTES, IL-6, and CCR2 expression by PTHrP (1–36) was dependent, at least in part, on both ERK and NF-κB activation in MCT cells. This further supports the notion that ERK activation is an important step in the signaling pathways leading to NF-κB-related inflammation.[30,31,38] Collectively, our findings strongly suggest that these intracellular pathways are involved in the mechanisms whereby PTHrP can promote renal inflammation.

A novel finding of this study was that PTHrP (1–36), apparently through the PTH1R, directly stimulated both MCP-1 production and cell migration in macrophage Raw 264.7 cells. Such interaction between both factors was recently suggested to occur in macrophages within human atheroma.[39]

In summary, we show here the critical role of PTHrP on the inflammatory process after kidney obstruction in mice. Our *in vitro* findings indicate that this protein upregulates several proinflammatory factors in tubuloepithelial cells and promotes monocyte/macrophage migration. Moreover, ERK-mediated NF-κB activation appears to be an important mechanism whereby PTHrP triggers renal inflammation. The use of rodent models has provided new insights into the molecular mechanisms underlying the pathophysiology of kidney obstruction,[8] although caution should be exercised in extending these experimental findings to humans. Considering this limitation, our present data suggest that PTHrP might be envisioned as a new inflammation marker and a potential therapeutic target in the obstructed kidney.

MATERIALS AND METHODS

Animal model of UUO

We used mice with or without renal PTHrP overexpression, as reported elsewhere.[26] Briefly, mice containing a human PTHrP (1–141) cDNA under control of a tetracycline operator were bred to mice with a construct consisting of a γ-glutamyl transpeptidase promoter fragment upstream of a tetracycline transactivator fusion protein to generate PTHrP-TG animals. This strategy allows the control of transgene expression.[6,26] The results with PTHrP-TG mice were compared with those obtained with control littermates (those bearing either one of the aforementioned constructs or normal CD-1 mice).[3,6] PTHrP overexpression in these mice does not induce significant changes in normal kidney morphology or basal renal function.[3,6,26] Experimental protocols were approved by the Institutional Animal Care and Use Committee at Fundación Jiménez Díaz.

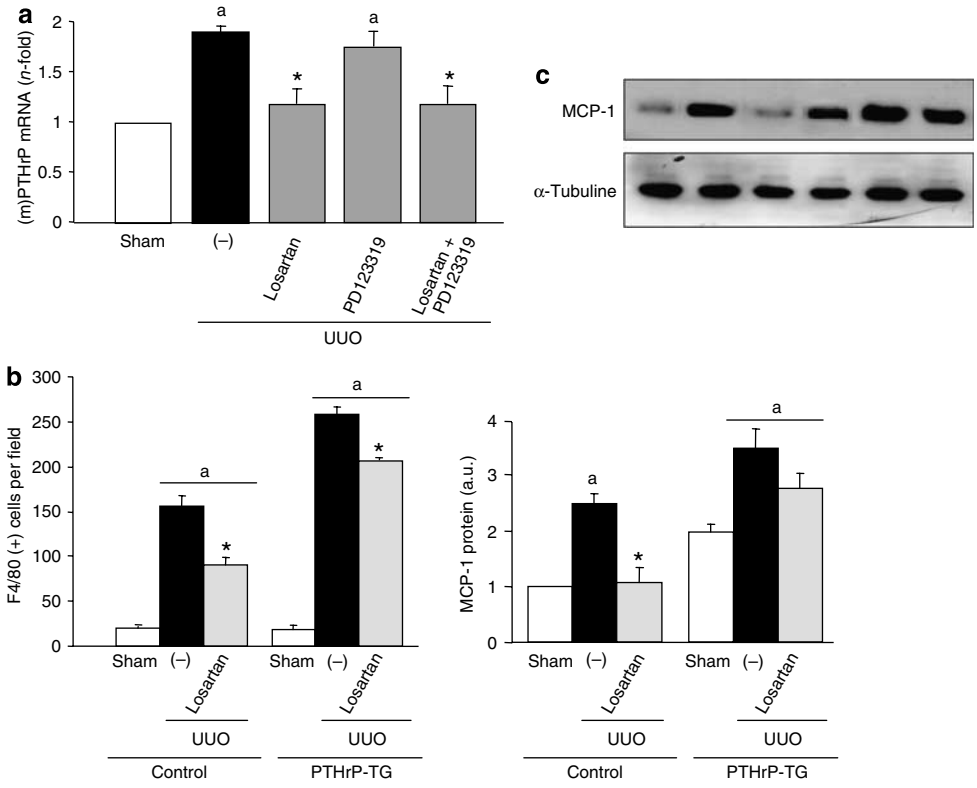

Figure 7 | Effect of Ang II antagonists on both PTHrP expression and proinflammatory alterations in the obstructed mouse kidney. (**a**) Mouse (m)PTHrP gene expression was analyzed by real-time PCR in the kidney of control mice at day 2 after UUO or sham operation. The number of renal interstitial macrophages (identified by F4/80 positivity) (**b**) and MCP-1 protein expression (shown a representative autoradiogram of western blot analysis) (**c**) were evaluated in the kidney of either PTHrP-TG mice or their control littermates at day 4 after UUO or sham operation. Losartan and PD123319 (10 and 30 mg kg^{-1} day^{-1}, respectively) were treated intraperitoneally 1 day before and also daily following UUO for the period studied in each case. Experimental values are mean ± s.e.m. of 4–6 animals per group. $^{a}P<0.01$ vs corresponding value in sham-operated mice. $^{*}P<0.01$ vs corresponding value in untreated mice. AU, arbitrary units.

UUO was performed under anesthesia in sex-unselected mice (weighing 25–35 g) by ligating the left ureter of each animal with 3-0 silk—at two locations and cutting between the ligatures—through an abdominal incision.[19] On different days thereafter, mice were killed and the obstructed and contralateral kidneys collected. Sham-operated mice, which had their ureters manipulated but not ligated, served as controls. A group of mice were treated with the PTH1R antagonist [Asn^{10}, Leu^{11}, D-Trp^{12}] PTHrP (7–34) amide [PTHrP (7–34)] (35 μg day^{-1}, intraperitoneally; Bachem, Bubendorf, Switzerland), the AT_1 receptor antagonist losartan (10 mg kg^{-1} day^{-1}, intraperitoneally; MSD, Merck Sharp & Dohme, Spain), or the AT_2 receptor antagonist PD123319 (30 mg kg^{-1} day^{-1}, subcutaneously; Sigma, St Louis, MO, USA), before UUO, and during the study period. These doses were found to be efficient to block some PTHrP or angiotensin II effects in mice.[19,27,37]

Kidney portions were separated from each mouse. One of them was fixed in 4% p-formaldehyde for histologic studies. One half of the remaining kidney portion was used for protein analysis and the other half for total RNA isolation, and they were stored at −20 °C for subsequent analysis.

Histology and immunohistochemistry

Fixed renal tissue sections were dehydrated and embedded in paraffin. Histological and immunostaining analysis were performed on serial paraffin-embedded renal tissue sections (2 μm) within the same mouse tissue. Renal histology was evaluated by hematoxylin/eosin and Masson's staining.

Immunohistochemistry was performed using a previously described protocol,[6] a rabbit polyclonal antibody to CD3 antigen in mouse T lymphocytes (Dako, Glostrup, Denmark) (at 1:200 dilution), and a monoclonal antibody against F4/80 antigen in murine monocytes/macrophages (Serotec, Oxford, UK).[6] Some tissue samples were incubated without the primary antibody as negative controls. All stainings were evaluated in at least eight different high-power fields per section in four sections from each mouse in a total of 4–6 mice per group. The percentage of stained area for Masson's staining was graded by the following semiquantitative score: 0, no staining; 1, up to 25%; 2, between 25 and 50%; and 3, >50%, as reported.[6,19] Tubular atrophy was graded from 0 to 3, on the basis of the presence of necrosis and brush border loss, irregular and thin epithelium, and/or thickened tubular basement membrane.[6,19] The number of CD3- and F4/80-positive interstitial cells was counted per field. All evaluations were performed by 2–3 independent observers in a blinded fashion, and the corresponding mean score value was obtained for each mouse.

Cell cultures

MCT cells (70 000 cells cm^{-2}) that respond to PTHrP (1–36)[6] and Raw 264.7 cells (ECACC 91062702) (3×10^{6} cells ml^{-1}), which have proven to be useful for studies on the mechanisms of

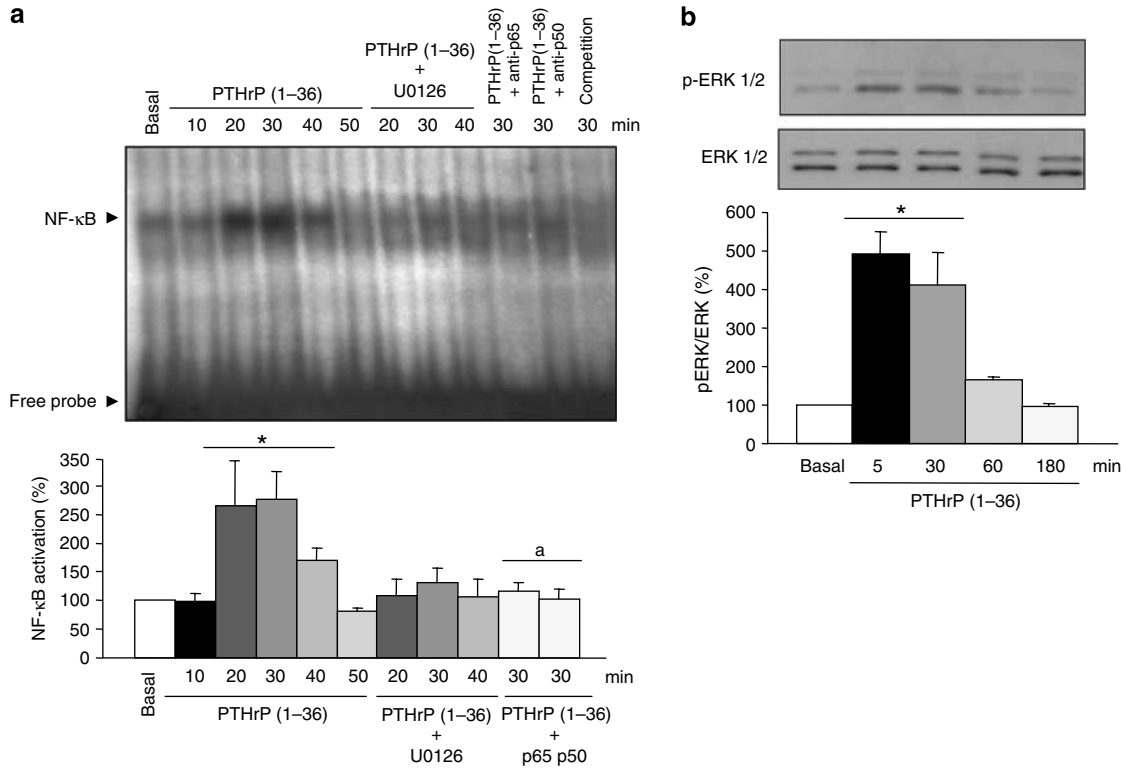

Figure 8 | PTHrP (1–36) induces NF-κB and ERK activation in tubuloepithelial MCT cells. (**a**) PTHrP (1–36), at 100 nM, induced NF-κB–DNA binding (analyzed by electrophoretic mobility shift assay (EMSA)) with a maximum at 20–30 min. Pretreatment with U0126 (25 μM) significantly reduced this effect of PTHrP (1–36), without affecting basal NF-κB activity (data not shown), in these cells. To characterize the proteins of NF-κB–DNA complex, in some experiments, nuclear extracts from MCT cells after stimulation with PTHrP (1–36) for 30 min were pre-incubated with an anti-p50 or anti-p65 antibody. As control for specificity of the binding reaction, these nuclear extracts were pre-incubated with a 100-fold excess of unlabeled NF-κB oligonucleotide. Densitometric values are mean ± s.e.m. from three independent experiments in duplicate**.** (**b**) PTHrP (1–36), at 100 nM, induced ERK phosphorylation in MCT cells. Western blot analysis was performed in MCT cell extracts using antibodies against pERK 1/2 or ERK 1/2. Basal = nonstimulated control (at 5 min). Relative densitometric values corresponding to pERK/ERK changes are mean ± s.e.m. from three independent measurements in duplicate. Representative autoradiograms for EMSA (**a**) and immunoblot analysis (**b**) are shown. *$P<0.01$ vs corresponding basal value (100%) and U0126-treated value. [a]$P<0.01$ vs corresponding PTHrP (1–36)-treated value at 30 min.

inflammation,[40–42] were incubated in serum-depleted medium (RPMI 1640), with or without PTHrP (1–36), for 5 min to 3 h. In some experiments, PTHrP (7–34) (1 μM) or several NF-κB inhibitors (parthenolide (25 μM; Sigma); carbobenzoxy-L-leucyl-L-leucyl-L-leucynal (MG-132) (10 μM; Calbiochem, San Diego, CA, USA); and Bay 11-7082 (25 μM; Sigma), or the ERK 1/2 kinase inhibitor U0126 (25 μM; Promega, Madison, WI, USA)) were added to cell cultures 1 h before PTHrP (1–36).

Macrophage migration assay

Subconfluent MCT cells were exposed to different agonists for 12 h. RAW 264.7 cells were seeded on Transwell chambers (5 μm pore size; Corning, New York, NY, USA) (2×10^5 cells per insert) and exposed to the medium conditioned by MCT cells, with or without PTHrP (7–34), or neutralizing rabbit polyclonal antibodies to either PTHrP (N-terminal C13 antiserum or C-terminal C6 antiserum)[43,44] or MCP-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (each at 1:100 dilution), for 24 h. To evaluate the direct effect of PTHrP (1–36) on Raw 264.7 cell migration, the MCT cell-conditioned medium was replaced by serum-depleted medium containing or not containing (basal medium) PTHrP (1–36), with or without PTHrP (7–34) or anti-MCP-1 antibody for 24 h. Preliminary experiments showed that this time was optimal to assess changes on Raw 264.7 cell motility. Cells migrating into the lower chamber were counted in a FACS Calibur cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis

Kidney tissue or cell samples were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonylfluoride (PMSF), and 0.8 μM aprotinin, pH 7.4). Proteins (30–60 μg per lane, as determined by Bradford's method (Pierce, Rockford, IL, USA), using bovine serum albumin (BSA) as standard) were separated on 10–15% polyacrylamide-sodium dodecyl sulfate gels under reducing conditions. Samples were then transferred onto polyvinylidene fluoride membranes (Millipore Corporation, Bedford, MA, USA), blocked with 5% defatted milk in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20, or 5% BSA in PBS with 0.05% Tween-20 (for PTHrP), and incubated overnight at 4 °C with the following rabbit polyclonal antibodies to MCP-1 (1:500; Santa Cruz Biotechnology); PTH1R (Ab-IV, 1:1000); PTHrP (C6, 1:2500);[3–6,39] ERK 1/2 or phosphorylated ERK 1/2 (Thr^{202}/Tyr^{204}) (Cell Signaling Technology; Beverly,

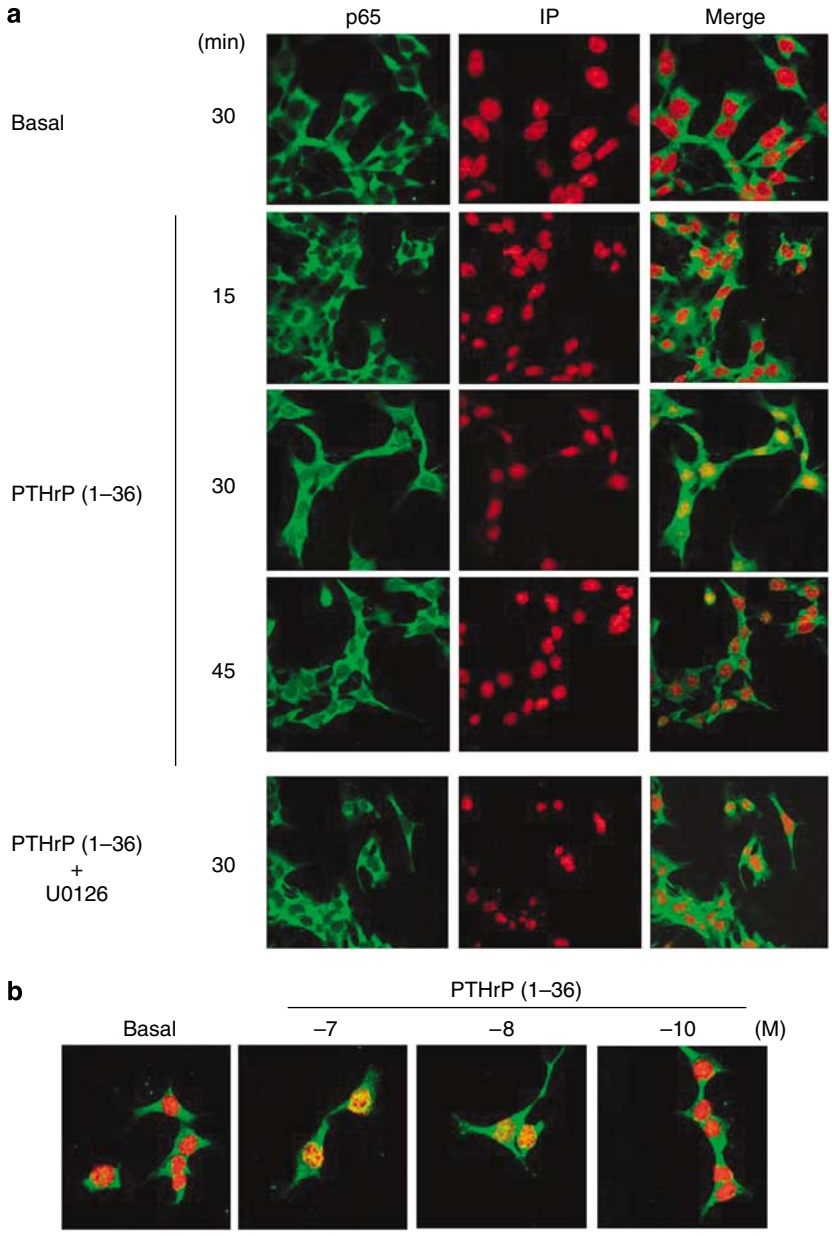

Figure 9 | PTHrP (1–36) induces p65 protein accumulation into the nucleus in MCT cells. Serum-depleted cells grown on multiwell chambers were untreated (basal) or treated with PTHrP (1–36) (100 nM) for different time periods (**a**) or with PTHrP (1–36) at different doses for 30 min (**b**). Double immunofluorescence staining was assessed, for the nucleus (with propidium iodide (IP); red) and using a fluorescein isothiocyanate-labeled IgG (green) for p65 detection. The overlaid images in red and green (merge) yielded an orange tone in the cell nucleus denoting the presence of intense nuclear p65 fluorescence in PTHrP (1–36)-treated MCT cells for 30 min. Pretreatment with the ERK inhibitor U0126 (25 μM) abolished this effect of PTHrP (1–36) (**a**). This represents the results of three independent experiments.

MA, USA) (each at 1:2000). Membranes were subsequently incubated with relevant peroxidase-conjugated goat anti-rabbit or anti-mouse IgG and developed by ECL chemiluminescence (Amersham, Buckinghamshire, UK). Densitometric values of fluorogram bands were normalized to those of β-actin or α-tubuline.

Real-time PCR

Cell total RNA was isolated with Trizol (Invitrogen, Groningen, The Netherlands), and gene expression was analyzed by real-time PCR using a described protocol and an ABI PRISM 7500 system (Applied Biosystems, Foster City, CA, USA).[19] Unlabeled mouse-specific primers for PTHrP (Mm00436057_m1); PTH1R (Mm00441046_m1); MCP-1 (Mm00441242_m1); RANTES (Mm01302428_m1); IL-6 (Mm00446190_m1); ICAM-1 (Mm00516023_m1); and CCR2 (Mm99999051_gH) and TaqMan MGB probes were obtained by Assay-by-Design^{SM} (Applied Biosystems). The mRNA copy numbers were calculated for each sample using the copy threshold value and normalized against 18S rRNA, as reported.[19]

Determination of NF-κB activity

NF-κB activity in renal and MCT cell extracts was assayed as described previously.[19,28] Briefly, ground-frozen kidney pieces were

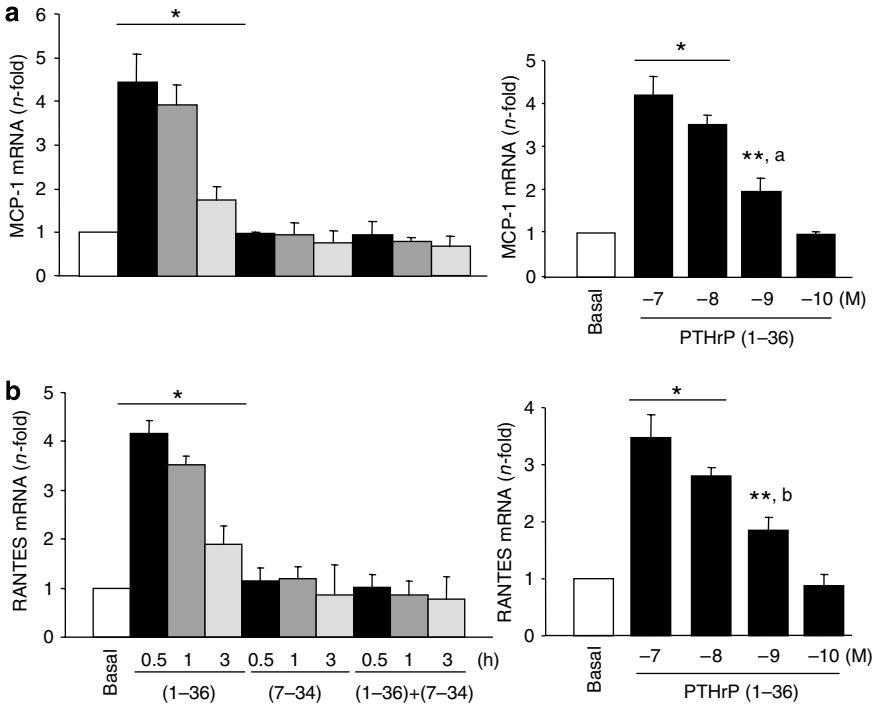

Figure 10 | PTHrP (1–36) time and dose dependently increases MCP-1 and RANTES gene expression in MCT cells. MCP-1 (**a**) and RANTES (**b**) mRNA levels were evaluated by real-time PCR. In some experiments, cells were pre-treated with PTHrP (7–34) (1 μM) for 1 h before addition of PTHrP (1–36) (100 nM) (**a** and **b**, left). Experimental values are mean ± s.e.m. from three independent experiments in duplicate. *$P<0.01$; **$P<0.05$ vs basal value. [a]$P<0.01$; [b]$P<005$ vs corresponding value at 10 nM PTHrP (1–36).

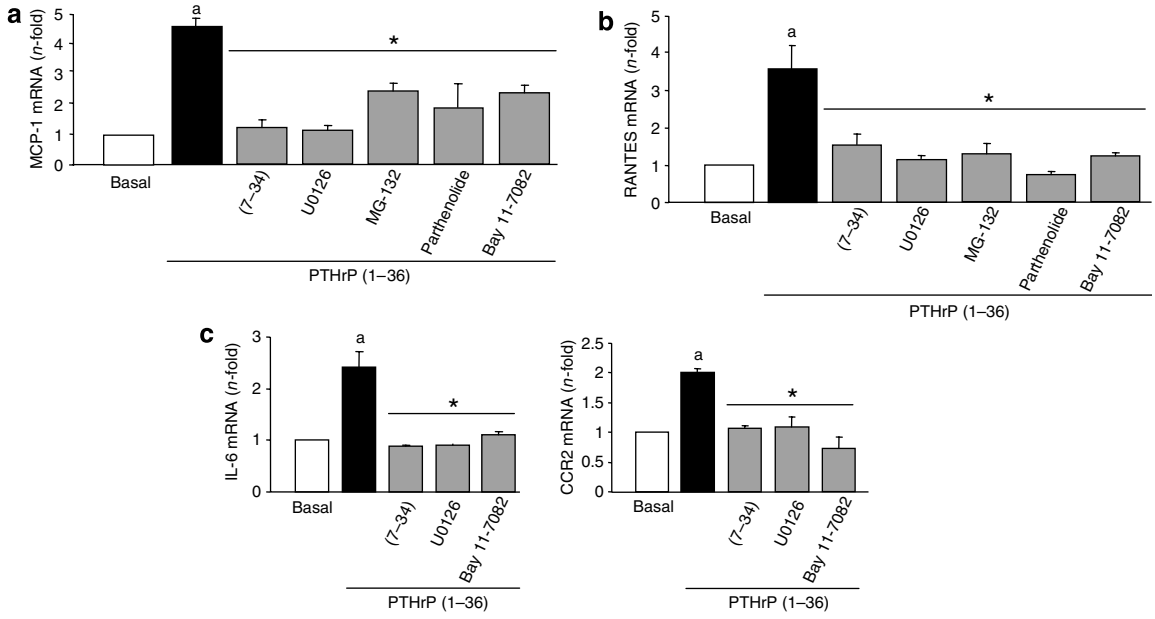

Figure 11 | Effect of different inhibitors on upregulation of proinflammatory factors induced by PTHrP (1–36) in MCT cells. Cells were treated with PTHrP (1–36) (100 nM) for 1 h. MCP-1 (**a**), RANTES (**b**), as well as IL-6 and CCR2 (**c**) mRNA levels were evaluated by real-time PCR. Different NF-κB inhibitors, MG-132 (10 μM), Bay 11-7082 (25 μM), and parthenolide (25 μM), the ERK inhibitor U0126 (25 μM), or PTHrP (7–34) (1 μM) were added 1 h before addition of the PTHrP peptide. Experimental values are mean ± s.e.m. from three independent experiments in duplicate. [a]$P<0.01$ vs basal value. *$P<0.01$ vs PTHrP (1–36) alone value.

resuspended in 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.6, 20% glycerol, 350 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.5 mM PMSF. After centrifugation at 40 000 *g* for 30 min at 4 °C, the supernatant was collected. MCT cells were lysed with 0.1% Triton in PBS. After centrifugation at 3000 *g* for 10 min, the pellet was resuspended in

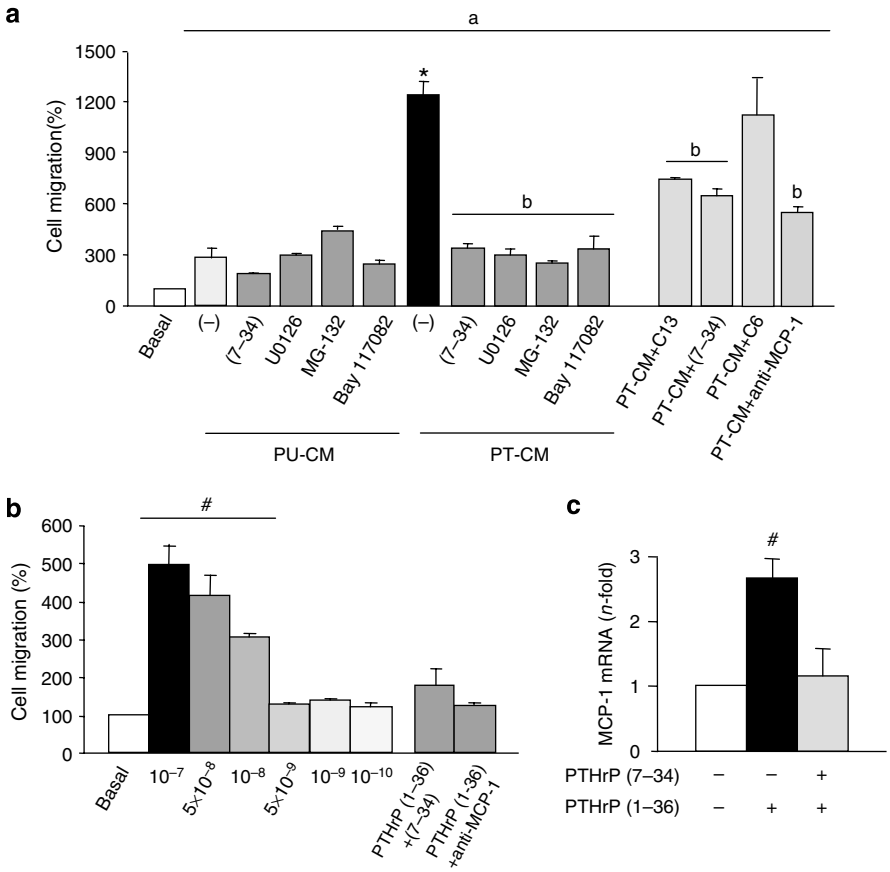

Figure 12 | PTHrP (1–36) induces migration of Raw 264.7 cells *in vitro*. (**a**) Serum-depleted MCT cells were exposed or not (basal) to PTHrP (1–36) (100 nM) with or without different inhibitors for 12 h. The resulting PTHrP (1–36)-treated or untreated cell-conditioned medium (PT-CM or PU-CM, respectively) was tested for chemoattractant activity on Raw 264.7 cells as described in the text. PTHrP (7–34) was used at 1 μM, and NF-κB inhibitors and U0126 were used at the same concentrations as in Figure 11. Addition of PTHrP (7–34) (1 μM) or neutralizing N-terminal anti-PTHrP antiserum C13, but not C-terminal anti-PTHrP antiserum C6 or pre-immune rabbit serum (data not shown), or an MCP-1 antibody (each at 1:100) to PT-CM significantly reduced Raw 264.7 cells' migration. Direct treatment of Raw 264.7 cells with PTHrP (1–36) (100 nM) stimulated these cells' migration (**b**) and MCP-1 gene expression (**c**); and both effects were inhibited by PTHrP (7–34). Moreover, the former effect was also abolished by an MCP-1 antibody. Experimental values are mean ± s.e.m. from at least three independent experiments in duplicate. $^{a}P<0.01$ vs basal medium value. $^{b}P<0.01$ vs PT-CM alone (–) value. $^{*}P<0.01$ vs PU-CM values, with or without (–) inhibitors. $^{\#}P<0.01$ vs basal value and values corresponding to PTHrP (1–36) at $<10^{-8}$ M or in the presence of different inhibitors.

20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA (ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid), 0.2 mM phenylmethylsulphonylfluoride (PMSF), and 1.4 μM aprotinin (nuclear extract). Tissue extracts (80 μg protein) and nuclear extracts (4 μg protein) were incubated with a ^{32}P-labeled NF-κB consensus oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′; Promega). Protein–DNA complexes were resolved on native 5% polyacrilamide/0.25 × TBE gels, and dried gels were exposed to radiosensitive film.[19,28] As specificity controls, some tissue and cell extracts were pre-incubated with a 100-fold excess of unlabeled NF-κB oligonucleotide. To characterize the NF-κB–DNA complex, nuclear extracts were pre-incubated for 2 h at 4 °C with 2 μl of anti-p50 or anti-p65 antibodies (Santa Cruz Biotechnology).[28]

To assess changes in p65 localization, subconfluent MCT cells on coverslips in multiwell plates were stimulated with PTHrP (1–36) (100 nM), with or without U0126 (25 μM), for 15–45 min. Cells were fixed with 64% isopropanol/15% polyoxyethylene (Cell-fixx; Shandon, Pittsburgh, PA, USA) and permeabilized with 0.2% Triton X-100 in PBS for 10 min. After blocking with 3% BSA for 30 min, cells were incubated with anti-p65 antibody (1:50; Santa Cruz Biotechnology) for 1 h, and then with fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody (1:200; Sigma). Samples were counterstained with propidium iodide to detect cell nuclei. Immunofluorescence analysis was performed with a Leica DM-IRB confocal microscope.

Statistical analysis

Results are expressed as mean ± s.e.m. The effect of UUO on different factors *in vivo* and both time course and dose–response of PTHrP (1–36) treatment *in vitro* were evaluated by analysis of variance followed by Dunnett's test. Mann–Whitney test was performed to analyze the differences between PTHrP-TG mice and their control littermates and the effect of different antagonists *in vivo*, as well as the effects of the different inhibitors *in vitro*. $P<0.05$ was considered significant.

DISCLOSURE

None of the authors have any relationships with companies that may have a financial interest in the content of this manuscript or any other interest to disclose.

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